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THE PROBLEM OF THE ANTIBODY NATURE OF THE RHEUMATOID FACTOR

BY

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To Maria-Theresia

PREFACE

The present investigation was carried out in 1957—1961 at the Municipal Bacteriological Laboratory, Aurora Hospital, Helsinki.

I wish to express my deep gratitude to Odd Wager, M.D., head of the Laboratory. He suggested to me the subject of the investigation and taught me the basic principles of immunology. At every stage of the work he supported me with valuable suggestions, sound criticism and excellent laboratory facilities.

I am much obliged to Nils Saris, Lic. Phil., for his advice concerning protein chemistry and for performing the immunoelectrophoretic and some of the chemical analyses, to Harri Nevanlinna, M.D., head of the Finnish Red Cross Blood Service, for provision of valuable reagents, and to Olavi Mäkelä, M.D., for some inspiring discussions.

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The work was facilitated by the skilled technical assistance of Mrs. Mirja Hannula and Miss Maija Saari. The English manuscript was corrected by Miss Elvi Kaukokallio. I express to them my heartfelt gratitude for this help.

I take pleasure in thanking also my friends at the laboratory for pleasant discussions and valuable assistance.

I am highly indebted to Professor Colin MacLeod, and to Drs. Marion Waller, John Vaughan, and Pekka Halonen for the provision of valuable reagents.

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Kimmo Aho

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CHAPTER I

INTRODUCTION

The rheumatoid factor (RF) is defined as the substance in the blood of patients with rheumatoid arthritis and some related diseases which is responsible for the agglutination of sensitized sheep erythrocytes, latex particles in the presence of Fraction II (Cohn), etc., under appropriate conditions (28). The serology of the rheumatoid factor and other immunological phenomena connected with rheumatoid arthritis have been extensively reviewed (17, 39, 40, 53).

Without any doubt the RF is associated in some fundamental way with the pathogenesis of rheumatoid arthritis. Perhaps the most common opinion is that the RF is an auto-antibody against the patient's own (autogenous) gamma globulin which has been altered in some way so as to become auto-antigenic. According to Vaughan (39) the antibody hypothesis is favored by the following arguments:

1. The RF is a 19 S gamma globulin;

2. Differential absorption studies with rabbit globulin follow the immunochemical rules for the behavior of antibody with cross-reacting antigen (page 20);

3. Like classic antibody to any given antigen, there is individual variation in the proportion of (rheumatoid) factor of a given serum capable of cross species reactivity;

4. Rheumatoid sera may contain other substances that are reactive with the patient's own tissues (anti-nuclear antibodies, immunoconglutinins).

It can be added that the RF apparently is formed in plasma cells (32).

The rheumatoid factor accepts as its reactant partner antigen-antibody complexes irrespective of the antigen involved, or isolated

gamma globulin which has been treated by heat or some chemical agents. Whether the denaturing only enhances the efficiency of the laboratory methods to demonstrate the RF or whether some fundamental differences exist in the reactivity of the RF with denatured and with native gamma globulin has been a matter of controversy (6, 7, 11, 19, 39).

If the RF is an antibody against human gamma globulin there remains the question of how the gamma globulin can become auto-antigenic. It has been suggested that the »rheumatoid antibody» is directed against antigenic determinants which are hidden in the native state and exposed when the native proteins become unfolded or otherwise denatured either by specific interaction with antigenic surface or by non-specific aggregation by heat or other maltreatment. A further requirement is that these determinants are shared by gamma globulins from various animal species (12, 18, 33).

General criticism on auto-antibodies has been presented by Waksman (48). More specifically, the arguments against the antibody hypothesis of the RF can be summarized as follows:

1. It has been difficult to demonstrate a reaction of the RF with unaltered gamma globulin. On the other hand, the RF reacts readily with immune complexes deriving from various animal species. It is not easy to understand this kind of broad reactivity unless it is postulated that the RF is an anti-antibody which crosses the species barrier. Classical immunology does not know of such a phenomenon;

2. A strong argument has been that the RF reacts with immune antibodies but not with »natural» ones;

3. Low-agglutinating activity is often demonstrated in healthy subjects;

4. It is a widely accepted opinion that the RF appears in the circulation only late in the course of the disease. This differs from the behavior of usual antibodies;

5. The precipitin reaction between the RF and gamma globulin does not fulfill all the requirements of a specific antigen-antibody interaction;

6. The half-life time of gamma globulin injected into patients with positive rheumatoid agglutination reactions is not demonstrably shorter than in normal persons.

Concerning the arguments for the antibody hypothesis it can

be remarked that any substance produced by the organism's cells to »co-work» with autogenous gamma globulin (e.g., some complement-like factor) would be likely to behave like the RF even if it were not an antibody itself.

On the other hand, arguments 2 and 4 against the antibody hypothesis have lost much of their importance. It has been observed that the RF, being itself a 19 S gamma globulin, demands as its reactant partner a 7 S gamma globulin. Many of the »natural» agglutinins have been found to be of the 19 S variety. During the past few years, evidence has been presented (14, 45) that the appearance of the RF in the circulation is an early and not a late phenomenon, as has been suggested earlier.

CHAPTER II

THE PROBLEMS AND THE OBJECT OF THE PRESENT WORK

Originally the object of the present work was a quantitative evaluation of the interaction of the rheumatoid factor (RF) with various specific precipitates. Soon it became apparent that employment of the micro-Kjeldahl technique in determining the nitrogen increase in precipitates and the use of sheep erythrocytes sensitized with homologous rabbit amboceptor (Waaler-Rose test) for demonstrating the RF remaining in the supernatants would not have led to any clearcut solution of the problems.

The first step forward was to use additional methods for measuring the RF. This soon directed the investigation to the theory of cross reaction and to the question of the possible antibody nature of the RF. Therefore the work of Maurer (31) on the cross reactions of gamma globulins from different species was reinvestigated and somewhat supplemented. The results were compared with the behavior of the RF. The concept of cross reactivity also required examination of a large number of rheumatoid sera using different tests.

It was felt that the elution of the RF from specific antigen-antibody complexes might be a way to demonstrate its adsorption when other methods had failed to give clearcut results. Further, the use of absorptions alone was not the ideal means to study the various reactive components of the RF. The endeavor therefore was to develop effective yet gentle elution methods.

In addition to ordinary class 7 S gamma globulins, it has been postulated that antigen-antibody complexes or antibodies denatured in serological reactions are antigens to the »rheumatoid antibody». However, no valid demonstration of the production by experimental animals of such »anti-antibodies» had been presented.

In this way the questions became centered around the problem of the antibody nature of the rheumatoid factor. The object was to set up experiments that might produce data for and/or against it. The main questions for which solutions were sought were:

1. What is the primary reactant partner of the RF? (If the RF is an antibody, what is the antigen?)

2. Does the reaction of the rheumatoid factor with its reactants resemble in all respects known antigen-antibody reactions with cross-reacting components?

3. Is it possible to produce »anti-antibodies» in experimental animals by means of well-defined antigens?

CHAPTER III

COMPARISON OF VARIOUS METHODS TO DEMONSTRATE THE RHEUMATOID FACTOR

Much work has been done in correlating the results given by different rheumatoid serological tests, (e.g., 49, 52). In most instances the results have been said to be in good agreement with each other, although exceptions from the rule have been presented.

In an earlier work of the author the serological findings in a series composed of 539 normal persons collected by random sampling and of 408 hospital patients with definite rheumatoid arthritis were analyzed (2, 47). There were 98 persons with a positive latex one-tube test and a negative Waaler-Rose test, but only 11 persons with a positive Waaler-Rose test and a negative latex one-tube test. This same pattern of greater sensitivity (and also lower specificity) of the latex test as compared with the Waaler-Rose test has emerged in all kinds of sera sent to the laboratory for rheumatoid serological tests. During three years the author has made readings from more than ten thousand serum samples.

Since negative results in the latex test (reactant of human origin) and, at the same time, positive results in the Waaler-Rose test (reactant of rabbit origin) were not in agreement with the antibody hypothesis of the rheumatoid factor, the nature of such sera was studied in more detail. For analysis, 1453 consecutive serum samples were taken. Further, the results given by the Waaler-Rose test and the SHC test (cf. below) were compared.

AGGLUTINATION TECHNIQUES

SHC Test (Agglutination of Sensitized Human O Rh Positive Cells)

Two different anti-D sera were used: »anti-D Ripley», an anti-D serum rendering O Rh positive cells agglutinable by most rheumatoid sera (this serum was obtained through the courtesy of Drs. Marion Waller and

J. Vaughan) and »anti-D 201/60», a serum suitable for Gm (a) typing. For coating the cells with anti-D antibodies, one part of packed cells, one part of anti-D serum and twelve parts of saline were used. The microscopic technique of Harboe and Lundevall (24) was followed. The dilution series always included as control the first serum dilution and non-sensitized cells. Differing from the original method, the reagents were first measured into 100 mm × 12 mm test tubes. Following incubation for one hour at room temperature, one drop of each mixture was placed within the rings of a glass slide. The readings were made immediately thereafter microscopically under low magnification. Unless otherwise stated, sera were not inactivated in the SHC test.

Agglutination of Latex Particles

The latex one-tube test was made as described by Singer and Plotz (37) using patients' own gamma globulin. The serum under test was fractionated with ammonium sulphate. The redissolved precipitate containing the RF also served as a source of the reactant. The final dilution of the rheumatoid serum in the one-tube test was 1:100. The titer determinations were made according to modification »*1.64 M ammonium sulphate fractionation of rheumatoid arthritis serum using latex-commercial gamma globulin*» (37). According to this modification, a dilution series was made from the redissolved precipitate. Into each tube 250 μg of Kabi gamma globulin (cf. page 21) was added.

The sera were inactivated by heating for 30 minutes in a water bath at 56°C. Polystyrene latex particles were obtained commercially from Difco Laboratories (»Bacto-Latex 0.81»). Using the one-tube technique, positive results were obtained in 4.1 per cent of normal controls and in 85 per cent of hospital patients with definite rheumatoid arthritis (2, 47).

Waaler-Rose Test (Agglutination of Sheep Cells Sensitized with Rabbit Amboceptor)[1]

Reagents. — Anti-sheep cell amboceptor was produced by a standard method (42). To make the final amboceptor four individual rabbit sera (Nos. 48—51) were pooled. Preliminary experiments had shown that each one of the four sera possessed an equal capacity to sensitize the sheep erythrocytes for the rheumatoid agglutination. The amboceptor was stored deep-frozen, divided into portions of one milliliter.

Sheep blood was drawn aseptically into isotonic glucose-citrate solution. Preferentially red cells from the same animal were used. The cells were stored in a refrigerator for not longer than two weeks. Before use they were washed three times in a large amount of saline.

Standard serum was a pool collected from several patients with rheumatoid arthritis. It was also stored deep-frozen and a small amount at a time was taken into use. Based on several simultaneous and consecutive determi-

[1] In this investigation the term Waaler-Rose test is reserved for agglutination of sheep cells sensitized with rabbit anti-sheep amboceptor.

nations its titer value was defined as 1400. All titers given by patient sera were adjusted according to the standard.

All dilutions were made in 0.85 per cent NaCl solution.

Amboceptor titration was made simultaneously with the titrations of the test sera. The dilution ratio between two consecutive tubes was $\sqrt[3]{2}$. Within the limits of experimental error the agglutination titer of the amboceptor has remained constant during three years.

Absorption of normal agglutinins was carried out by adding to 0.2 ml of serum 3.0 ml of saline and 0.1 ml of packed sheep erythrocytes. The mixture was kept in the water bath at 37°C for one hour and in the refrigerator overnight. In about one of a thousand sera examined this absorption has not been sufficient for removal of normal agglutinins. In most instances these exceptional sera have been from patients with systemic lupus erythematosus.

Titration of the serum under test was made by measuring different amounts of the absorbed serum dilution, using pipettes of different sizes and then filling the tubes to a constant volume of 0.5 ml. The dilutions in the tubes were separated by a factor of 2. This method was found to give smaller standard deviation values in parallel determinations than the serial twofold transfer dilution method. Moreover, it gives a truer value, whereas in the serial transfer method the error is progressively greater in the higher dilutions (unless every step is made with a new pipette). The standard serum was titrated more accurately, the dilution ratio between two successive tubes being only $\sqrt{2}$. Where possible, semiautomatic pipettes were used (B—D Cornwall Luer-Lok Syringe No. 1250, Becton, Dickinson and Co., Rutherford, N.J.)

The sensitized sheep cell suspension was made by mixing equal amounts of 1 per cent suspension of washed cells and amboceptor dilution corresponding to $\frac{1}{3}$ of the minimum agglutinating dose ($\frac{1}{3}$ MAD), if not otherwise stated. After about 15 minutes, 0.5 ml of sensitized sheep cell suspension per tube was added to the dilution series under test. Thus the total volume in each tube was 1.0 milliliter and the concentration of the red cell suspension 0.25 per cent, calculated from the final volume.

The use of $\frac{1}{3}$ MAD was a compromise between sensitivity and specificity. In an unpublished series of about 300 sera titrated with two or three different sensitizing doses it had been demonstrated that $\frac{1}{2}$ MAD was very near to the limit where some apparently normal sera tend to cause agglutination. On the other hand, a notable number of positive rheumatoid sera is missed if a sensitizing dose less than $\frac{1}{4}$ MAD is used. When the sensitizing dose was varied, the agglutination pattern differed somewhat from serum to serum.

The tubes were stored overnight in the refrigerator. Incubation of the tube series in a water bath of 37°C was omitted, as it had been found that the final result was not affected by this. Taking into account the agglutination experiments at different temperatures (table 3) it is apparent that preliminary incubation affords no advantages.

On the following morning the tubes were shaken carefully in their racks and allowed to stand for 3—5 minutes. The readings were made as bottom readings against the even light of a mercury vapor lamp. The maximum dilution was noted in which the serum was still capable of producing agglutination of sensitized cells. The final result was obtained by comparing this reading with that given by the standard serum and calculating the result accordingly.

Based on unpublished preliminary experiments, a titer less than 64 was regarded as negative, 64 as doubtfully positive and titers 128 or more as definitely positive. When the sera studied were divided into a positive and a negative group only, the titer of 64 was regarded as positive. Later it was shown that in a series of 489 persons (2) who most improbably suffered from rheumatoid arthritis, there were 1.6 per cent positive results if the titer 64 is accepted as the lowest positive value. Correspondingly there were 67 per cent positive results in a series of 408 hospital patients with definite rheumatoid arthritis (47). Observations made in these works suggested that titers lower than 64 in most instances probably were not due to the specific action of the RF. This was also suggested by the absorption pattern shown in table 6 in the present report: low-agglutinating activity of normal serum was not absorbable with specific precipitate. Similar absorption patterns have been observed also when using rabbit amboceptor and not only the guinea pig or horse amboceptors shown in the table.

In absorption and elution experiments the serial twofold transfer dilution method was used, because the starting dilution of the series varied from experiment to experiment and the dilution series were in most instances too long for the above described method. When possible, dilutions were made in bulk and then distributed into appropriate tubes. The results were read both as bottom readings and by reading each tube separately against a desk lamp.

Titers 256, 512, 1024, etc., indicate that the dilution series were made by using serial transfer dilutions. If all possible precautions are taken into consideration, they may represent the true values. On the other hand, titers 250, 500, 1000, etc., indicate that different amounts of prediluted serum were measured into each tube, as previously described. These approximate values were used because the measurements carried out with the semiautomatic pipettes do not justify greater accuracy.

All titers are expressed as reciprocals of dilutions, calculated from the total volume of reagents in the tube.

RESULTS

Of the 1453 consecutive serum samples sent for rheumatoid serological tests, the Waaler-Rose was positive in 402 cases and the latex one-tube test in 570 cases. There were 18 cases with obvious disparity between the results yielded by the two tests (i.e., Waaler-Rose was doubtfully positive and latex negative, or

Waaler-Rose was definitely positive and latex negative or doubtfully positive).

From these 18 sera, titer determinations were made with the latex technique and the SHC (Ripley) test, both from inactivated serum. The results are presented in table 1.

TABLE 1

SEROLOGICAL RESULTS IN 18 CASES WITH DISPARITY BETWEEN RESULTS GIVEN BY WAALER-ROSE AND LATEX ONE-TUBE TESTS

Serum No.	Waaler-Rose Titer	Latex One-Tube	Latex Titer	SHC (Ripley) Titer
81	250	—	5120	<20
532	64	—	1280	160
639	250	—	2560	<20
674	128	—	320	40
700	64	—	320	<20
706	250	—	640	not done
716	250	—	640	160
758	64	—	160	not done
775	250	+?	2560	80
786	128	—	2560	<20
791	500	+?	2560	<20
885	128	—	5120	<20
1037	500	+?	5120	<20
1101	250	—	2560	<20
1178	64	—	320	<20
1276	64	—	2560	not done
1300	128	+?	640	40
1358	64	—	2560	40

All the 18 sera, negative or only doubtfully positive in the latex one-tube test, showed at least some degree of RF activity when using the titer determination [1]. In 16 out of the 18 sera there was a prozone in spite of the ammonium sulphate fractionation. Six of the 15 sera studied were positive in the SHC (Ripley) test.

The relationship between the SHC and Waaler-Rose tests in a series of consecutive serum samples is illustrated in table 2. In order to save reagents, the SHC tests were performed with only one serum dilution. For the same reason, separate control and

[1] In an unpublished series of 400 sera it had been found that the latex one-tube test was a distinctly more sensitive means for demonstrating the rheumatoid factor than the titer technique if titer value 320 is accepted as the lowest positive result.

TABLE 2

COMPARISON OF WAALER-ROSE AND SHC TESTS

Waaler-Rose	SHC (201/60)	SHC (Ripley)	No. of Sera
+	+	+	5
+	+	—	0
+	—	+	62
—	+	+	3
+	—	—	4
—	+	—	0
—	—	+	20
—	—	—	130
71	8	90	224

rheumatoid series were not studied. The sera were diluted 1 : 10, after which an equal amount of sensitized cells was added, making the final dilution of 1 : 20.

It is seen from the table 2 that the SHC (201/60) test gave only a few positive results, and all these cases were also positive in the SHC (Ripley) test. Of the eight positive sera in the SHC (201/60) test, three were Waaler-Rose negative. The SHC (Ripley) test gave a considerably larger number of positive results than the Waaler-Rose test. Twenty of the 23 cases positive in the SHC (Ripley) test but negative in the Waaler-Rose test were patients suffering from rheumatoid arthritis (10 cases), or this was at least one of the possible diagnoses (10 cases). In the three remaining cases there was nothing indicative of rheumatoid arthritis. In all of the four cases where the Waaler-Rose test was positive and the SHC (Ripley) test negative the Waaler-Rose titer was 64.

CONCLUSIONS

Practically all the cases with a positive Waaler-Rose test were positive also in the latex one-tube test, but not *vice versa*. The few negative results were probably due to the prozone phenomenon. The SHC (Ripley) test also gave more positive results but probably also more false positives than the Waaler-Rose test (if titer 20 is accepted as the lowest positive value in the SHC test). It appears that the use of reactant of human origin is a more sensitive method for demonstrating the RF activity than the use of rabbit amboceptor.

CHAPTER IV

ADSORPTION OF THE RHEUMATOID FACTOR TO AND ELUTION FROM ANTIGEN-ANTIBODY COMPLEXES

Wager (43) was the first to absorb away the RF activity from the serum using sensitized sheep erythrocytes. He was also successful in eluating the factor from sensitized cells in a saline solution at 56°C. More recently, Heimer *et al.* (25) used sensitized sheep stromata for absorption and eluated the factor by changing the pH. This method was claimed by the authors to be suitable for preparative purposes, although the consumption of reagents was great. The observation of Heller and coworkers (26) was the first to suggest the heterogeneity of the RF. By absorbing rheumatoid serum with sensitized sheep cells, the reaction with F II-treated tanned erythrocytes remained nearly unaltered.

The following antigen-antibody precipitates have been studied for their reactivity with the RF (11, 13, 38):

— Egg albumin — rabbit anti-egg albumin
— Conalbumin — rabbit anti-conalbumin
— Diphtheria toxin — human antitoxin
— Egg albumin — guinea pig anti-egg albumin
— Egg albumin — horse anti-egg albumin
— Human albumin — rabbit anti-human albumin
— Human gamma globulin — rabbit anti-human gamma globulin
— Pneumococcus type III polysaccharide — horse anti-pneumococcus type III polysaccharide.

All complexes containing rabbit antibodies were found to be reactive with the RF. Besides a measurable nitrogen increase in the precipitates, they could absorb all the RF activity measurable by the Waaler-Rose test, but not that measurable by the SHC test. No reactivity of the RF with precipitates containing the antibody deriving from other animal species could be demonstrated.

Vaughan (28) stated that he had been successful in his attempts to absorb the RF with specific precipitates prepared from horse and guinea pig antisera, but he has given no further details.

In order to get more information on the specificity of the RF, its reactivity with various antigen-antibody complexes was investigated in experiments described in this chapter. After absorption, the supernatant RF activity was tested using reactants from different animal species. Attempts were made to eluate the RF activity from antigen-antibody complexes. This was done not only in order to exclude the possibility of a non-specific inactivation of the RF, but also to obtain a clearer view of the different reactive components of the RF.

MATERIAL AND METHODS

Reagents. — Egg albumin (Ea) was a commercial five times crystallized product (lot 4911) of Pentex Inc., Kankakee, Ill. Human gamma globulin (HGG) was obtained commercially from AB. Kabi, Stockholm as a 12 per cent solution. Pneumococcus type I polysaccharide was obtained through the courtesy of Professor Colin MacLeod, New York. Diphtheria toxoid (lot 31/c), a highly purified product containing 1800 Lf/ml, and horse diphtheria antitoxin containing 1200 Lf/ml were obtained through the courtesy of Dr. P. Halonen of Lääketehdas Orion, Pharmaceutical Manufacturers, Helsinki. Horse anti-sheep cell amboceptor was a commercial product from Burroughs Wellcome & Co., and it was obtained through the courtesy of Winthrop Products Ltd.'s representative in Finland. »Merthiolate» (trade-mark of Eli Lilly and Co. for Thimerosal) 1:5000 was used as preservative for sera.

Production of Immune Sera. — The production of human diphtheria antitoxin has been described elsewhere (3). In short, each of two rheumatoid subjects belonging to the serum group Gm (a+) received in all six injections, a total of about 150 Lf of alum-precipitated toxoid. The injections were given by the intramuscular route once a week.

Rabbit anti-egg albumin was a pool of the sera of eight rabbits having received alum-precipitated Ea for five consecutive weeks. Each rabbit received a total of 20 mg Ea distributed over 16 injections. In some cases this was not sufficient, and then 8 additional injections were given.

Rabbit diphtheria antitoxin was prepared by giving each of five rabbits a total of 1500 Lf of diphtheria toxoid distributed over 17 injections. The first injection was given subcutaneously with Freund incomplete adjuvant, followed by 8 intravenous and 8 subcutaneous injections of toxoid in saline solution.

Rabbit anti-pneumococcus type I polysaccharide was obtained by giving three rabbits washed pneumococcal type I saline suspension intravenously

according to instructions from Professor Colin MacLeod. Only one rabbit produced sizable amounts of antibodies, and from it only a small quantity of blood was obtained.

Guinea pig diphtheria antitoxin was produced by giving each of 29 guinea pigs a single dose of 72 Lf of diphtheria toxoid intraperitoneally with Freund incomplete adjuvant. Three weeks later the same amount of toxoid in saline solution was given subcutaneously. Two weeks thereafter the guinea pigs were bled and the sera were pooled.

Guinea pig anti-sheep cell amboceptor was made in ten individual guinea pigs, as described by Wager (43). The sera were stored separately.

Agglutination Reactions. — The techniques for the Waaler-Rose, SHC, and latex tests have been described in the technical section of chapter III. The method of agglutination of sheep cells sensitized with guinea pig or horse amboceptors was the same as in the Waaler-Rose test. When the latex test was made from eluates, the ammonium sulphate fractionation was omitted.

Preparation of Specific Precipitates. — The approximate equivalence point was determined by preliminary precipitin tests, as described by Kabat and Mayer (27). In most instances a constant volume of serum was added to varying quantities of antigen, increasing in twofold steps. After removing the precipitate, the supernatant was tested for antibody and/or antigen excess using capillary tubes. Diphtheria antitoxin assays were also made in some instances in guinea pig skin (15).

The antigen-antibody mixture was incubated in the water bath at 37°C for one hour and kept overnight in the refrigerator. In most instances large amounts of specific precipitate were prepared to serve as a constant source of material. The precipitates were spun down, washed three times with a large amount of chilled saline, and suspended in saline. As preservative, »Merthiolate» 1:5000 was added to the precipitate suspension.

Absorption of the RF onto Specific Precipitates. — Either packed precipitate or precipitate suspension was mixed with undiluted rheumatoid serum. Unless otherwise indicated, the mixture was kept overnight in the refrigerator. If more than one absorption was made, packed precipitate from the second absorption onwards always was used. Before performance of the Waaler-Rose and latex tests with absorbed sera, portions of the sera were inactivated by keeping them in a water bath at 56°C for 30 minutes. Non-inactivated portions were employed in the SHC test.

Elution of the RF from Specific Precipitates. — The precipitate with adsorbed rheumatoid factor was washed three times with a large volume of chilled saline. The saline from the third washing showed in some instances a slight latex agglutinating activity (titers 40—80) whereas Waaler-Rose and SHC tests were consistently negative. Some spontaneous precipitate formation was seen in absorption control tubes of sera K.O. and H.K. containing rheumatoid serum alone, diluted 1:2 with saline. Eluate made of the spontaneous precipitate K.O. gave a Waaler-Rose titer of 16 and a latex titer of 160 but no measurable activity in the SHC test. The eluate

made of the minute spontaneous precipitate H.K. yielded no measurable RF activity whatsoever.

One and a half milliliters of an acetate buffer pH 5.0 was added to 95—210 μg N of the washed precipitate, onto which the RF from one milliliter of serum had been adsorbed. The suspension was kept 1—1.5 hours at room temperature and occasionally agitated gently by hand. The precipitate was spun down and the supernatant separated. The whole procedure was repeated. The two supernatants were combined, neutralized with a slight excess of tris (hydroxymethyl) aminomethane (Sigma Chemical Co.), and adjusted to ionic strength 0.15 with distilled water. This resulted in a final volume four times that of the original rheumatoid serum used for adsorption. Eluates were not inactivated before performance of the Waaler-Rose and latex tests.

Titers of the eluates were expressed in terms of the original volume of serum used for adsorption.

Rheumatoid Sera. — Sera of seven different patients were employed. Two of the sera originated from subjects who had been immunized with diphtheria toxoid (A.S. and H.K.). Two sera (V.I. and T.A.) were selected because of ability to agglutinate O Rh positive cells sensitized with anti-D 201/60. In serum K.O. there was an exceptionally large amount of RF. The last two sera (S.K. and A.I.) were high-titered rheumatoid sera. These seven sera were not inactivated and they were stored deep-frozen.

Moreover, a pooled rheumatoid serum (R-pool) was used in many experiments. It was composed of ten individual rheumatoid sera. It was inactivated and the normal agglutinins to sheep erythrocytes were removed by absorption.

Normal Sera. — Commercial pooled serum (N-pool) obtained from the Finnish Red Cross Blood Service was used.

Nitrogen Analyses. — The Markham modification (30) of the micro-Kjeldahl method was followed, using a Scholander burette. Unless otherwise stated, determinations were made in duplicate.

RESULTS

A. EXPERIMENTS TO DEVELOP METHODS FOR ELUATING THE RHEUMATOID FACTOR AWAY FROM ANTIGEN-ANTIBODY COMPLEXES

Elution of the RF from specific precipitates was tried by varying the temperature, the ionic concentration or the pH. Experiments were carried out with the Ea — rabbit anti-Ea system. The eluates were tested for RF activity with Waaler-Rose and latex techniques.

The rheumatoid serum (R-pool) was treated with an amount of precipitate just sufficient to remove all the RF activity measurable with the Waaler-Rose test. It was felt that the use of

RF in great excess would have led to selection and therefore to incomplete conclusions as to the specificity of the eluates. The precipitates were incubated with rheumatoid serum overnight in the refrigerator. On the following morning the precipitates were washed three times with a large amount of chilled saline. They were then submitted to various elution procedures.

Of the various methods tried, incubation of the precipitate suspension at 56°C for ten minutes did not yield measurable amounts of RF activity in the supernatant. Treatment of the precipitate with a 9 per cent NaCl solution gave small amounts of RF activity measurable with the latex technique but not with the Waaler-Rose test. Varying the pH was found to be a suitable method. Details of this technique are given in the technical section.

Using this method, under optimal conditions (i.e., using moderate excess of RF) it was possible, as shown below, to recover in eluates about one-fourth of the RF activity subjected to adsorption.

Elution of the RF from sensitized cells onto which it had been adsorbed was tried only by incubation at elevated temperatures.

First, the influence of temperature on the agglutination reactions was studied. R-pool was used in the Waaler-Rose test, whereas the SHC test was performed with two anti-D sera and two rheumatoid sera (V.I. and T.A.). Anti-D Ripley and rheumatoid serum T.A. gave higher titers than anti-D 201/60 and rheumatoid serum V.I., but the reaction pattern at various temperatures always was similar. The results of the Waaler-Rose test were read after 1 hour, 3 hours, and 18 hours, and the results in the SHC test after 1 hour and 3 hours. The reaction was somewhat slower at lower temperatures. Some of the readings at 3 hours are illustrated in table 3.

TABLE 3

WAALER-ROSE AND SHC ACTIVITIES AT DIFFERENT TEMPERATURES

Temperature	Waaler-Rose Titer (R-pool)	SHC (Ripley) Titer (Serum V.I.)
4°C	250	2560
22°C	500	2560
37°C	250 [1]	5120
45°C	<32	2560

[1] Weak agglutination pattern.

It is observed that the SHC agglutination took place very well at 45°C, whereas the Waaler-Rose agglutination diminished already at 37°C. That this evidently was not due to dissociation of the bond between the sheep cells and the sensitizing amboceptor was suggested by the behavior of the amboceptor under similar conditions. Regardless of changes in temperature, its agglutination titer for sheep cells remained the same.

As no agglutination of sheep cells took place at 45°C, this temperature was tried in the elution experiments. As seen below (page 32), RF activity was demonstrable in the eluate after incubation of the cells for one hour at 45°C, and much of the hemolysis seen at 56°C was avoided.

B. SPECIFICITY OF THE RHEUMATOID FACTOR ADSORBED ONTO AND ELUATED FROM SPECIFIC PRECIPITATES

The reaction of rheumatoid serum (R-pool) with Ea — rabbit anti-Ea precipitates prepared in slight antibody or antigen excess is demonstrated in table 4.

TABLE 4

REACTION OF RHEUMATOID FACTOR WITH EA—RABBIT ANTI-EA PRECIPITATES MADE IN SLIGHT ANTIBODY OR ANTIGEN EXCESS

Precipitate Made in	Volume of Precipitate Suspension Used	μg N in Precipitate after Treatment with 1 ml of		μg N Absorbed from R-pool by Difference	Waaler-Rose Titer of Supernatant (½ MAD)
		N-pool	R-pool		
Ab excess	0.5 ml	67	87	20	64
»	1.0 ml	133	154	21	32
»	2.0 ml	265	297	32	<16
Ag excess	0.5 ml	76	94	18	16
»	1.0 ml	145	173	28	<16
»	2.0 ml	277	321	44	<16

Waaler-Rose titer of non-absorbed R-pool was 1024.

It can be seen that measurable amounts of nitrogen were adsorbed from the rheumatoid serum onto the precipitate. Further it can be observed that the adsorptive power of the precipitate for nitrogen and RF activity was to some degree better in the region of slight antigen excess than in antibody excess.

When a rheumatoid serum with an exceptionally high Waaler-Rose titer (K.O.) was adsorbed with Ea — rabbit anti-Ea precipi-

TABLE 5

ABSORPTION OF CONSTANT VOLUME OF RHEUMATOID SERUM WITH DECREASING AMOUNTS OF EA—RABBIT ANTI-EA PRECIPITATE

Amount of Precipitate Used	Cells Sensitized with	Dilution of Rheumatoid Serum									Aggl. Titer by Bottom Reading
		16	32	64	128	256	512	1024	2048	4096	
115 μg N	1/2 MAD	+	+	(+)	—	—	—	—	—	—	32
	1/4 »	(+)	—	—	—	—	—	—	—	—	<16
	1/8 »	—	—	—	—	—	—	—	—	—	<16
58 μg N	1/2 MAD	+ +	+ +	+ +	+ +	+	+	—	—	—	512
	1/4 »	(+)	—	—	—	—	—	—	—	—	<16
	1/8 »	—	—	—	—	—	—	—	—	—	<16
29 μg N	1/2 MAD	+ + +	+ + +	+ + +	+ +	+ +	+	(+)	—	—	512
	1/4 »	(+)	(+)	(+)	—	—	—	—	—	—	<16
	1/8 »	—	—	—	—	—	—	—	—	—	<16
15 μg N	1/2 MAD	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	—	—	1024
	1/4 »	+	+	(+)	(+)	—	—	—	—	—	32
	1/8 »	—	—	—	—	—	—	—	—	—	<16
8 μg N	1/2 MAD	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	(+)	—	1024
	1/4 »	+ +	+ +	+ +	+ +	(+)	—	—	—	—	128
	1/8 »	—	—	—	—	—	—	—	—	—	<16
4 μg N	1/2 MAD	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	(+)	—	1024
	1/4 »	+ +	+ +	+ +	+ +	+ +	—	—	—	—	256
	1/8 »	—	—	—	—	—	—	—	—	—	<16
None	1/2 MAD	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	(+)	—	1024
	1/4 »	+ + +	+ + +	+ + +	+ +	+ +	(+)	—	—	—	256
	1/8 »	+ +	+ +	+ +	+	—	—	—	—	—	128

tate, one absorption was not sufficient to remove all Waaler-Rose activity.

Also diphtheria toxoid — rabbit antitoxin precipitate was found to absorb definitely more nitrogen from rheumatoid sera than from normal ones. The amount of rabbit anti-pneumococcus type I polysaccharide available was not sufficient for quantitative studies. However, specific precipitate made from this serum also was found to remove all Waaler-Rose activity from rheumatoid serum.

Next, the effect of absorption with decreasing amounts of Ea — rabbit anti-Ea precipitate [1] of a constant volume of rheumatoid serum (R-pool) was studied.

It is seen from table 5 that very small amounts of specific precipitates were sufficient to remove the capacity of the rheumatoid serum to agglutinate weakly sensitized cells.

After absorption of the Waaler-Rose activity with Ea — rabbit anti-Ea precipitate, detailed analysis of the supernatant was made. R-pool and four individual rheumatoid sera (T.A., A.I., K.O., and S.K.) were used in these experiments.

No titer decrease or only a small decrease was observed when the supernatants were tested by the SHC or latex techniques. A definite decrease was seen but demonstrable amounts of activity were still present in the supernatants when sheep cells sensitized with guinea pig or horse anti-sheep amboceptor were used. The behavior of the R-pool with the latter two amboceptors and also with rabbit amboceptor is demonstated in table 6.

In addition to the decrease of titer shown in table 6, a definite weakening of the agglutination pattern also was seen.

The reaction of the RF with human specific precipitates has been described in detail elsewhere (3, 44). It was shown that the RF can be adsorbed to and eluated at pH 5 from the patient's own specific precipitate, consisting of diphtheria toxoid and autogenous antitoxin. Rheumatoid antitoxic precipitates also reacted with the RF of other, non-immunized patients. Table 7 illustrates some of the most essential absorption results.

As seen, repeated absorptions with autogenous (the antibody

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[1] In this experiment and in those described later, Ea—rabbit anti-Ea precipitate means a certain batch of precipitate made in slight antigen excess and containing 115 μg N per ml.

TABLE 6

REACTION OF NON-ABSORBED SERA AND SERA ABSORBED WITH EA—RABBIT ANTI-EA PRECIPITATE WITH SHEEP ERYTHROCYTES SENSITIZED WITH VARIOUS AMBOCEPTORS

Serum Under Test	Sheep Cells Sensitized with Amboceptor from	Sensitizing Dose of Amboceptor	Titer of Non-Absorbed Serum	Titer of Serum Absorbed with Rabbit Anti-Ea Precipitate
R-pool	rabbit	½ MAD	1024	<32
		¼ »	512	<32
N-pool	»	½ MAD	<32	<32
		¼ »	<32	<32
R-pool	guinea pig	½ MAD	256	128
		¼ »	32	<32
N-pool	»	½ MAD	32	32
		¼ »	<32	<32
R-pool	horse	½ MAD	512	128
		¼ »	32	<32
N-pool	»	½ MAD	32	32
		¼ »	<32	<32

TABLE 7

EFFECT OF ABSORPTIONS WITH RHEUMATOID ANTITOXIC PRECIPITATES ON THE RF ACTIVITY OF WHOLE RHEUMATOID SERUM AND OF RHEUMATOID COLD PRECIPITATE

	Rheumatoid Precipitate Used for Absorption [1]	Waaler-Rose Titer		Latex Titer		SHC (Ripley) Titer	
		Before Abs.	After Abs.	Before Abs.	After Abs.	Before Abs.	After Abs.
Rheumatoid serum A.S. ..	A.S.[2]	512	128	40960	320	10240	2560
Rheumatoid serum A.S. ..	H.K.[2]	512	32	40960	320	10240	<40
Cold precipitate A.S.	A.S.[3]	512	16	40960	160	5120	20
Cold precipitate K.O.	H.K.[2]	8192	16	≥327680	640	≥10240	20

[1] The whole sera were absorbed four times and the cold precipitates twice with specific precipitates.

[2] Total amount of precipitate nitrogen used for absorption was 828 μg.

[3] Total amount of precipitate nitrogen used for absorption was 414 μg.

in the precipitate and the RF originating from the same patient) or isogenous (the antibody in the precipitate and the RF originating from different patients) antitoxic precipitates did not completely remove the RF activity from the whole rheumatoid serum. However, when cold precipitates made of rheumatoid sera by dilution with distilled water (1:16) and redissolved in saline were used, the absorption was nearly complete. The behavior of the cold precipitate in absorption experiments carried out with rabbit specific precipitate did not differ from that of the whole serum, i.e., a strong agglutinating activity in the SHC and latex tests was seen in the supernatant after absorption with rabbit precipitate.

Diphtheria toxoid — guinea pig antitoxin precipitate made in slight antigen excess did not remove measurable amounts of nitrogen from the rheumatoid sera. One milliliter of rheumatoid serum S.K. absorbed with a given amount of precipitate gave a value of 149 μg N, the corresponding figures for R-pool and N-pool being 142 μg N and 148 μg N, respectively. When the supernatant after absorption with guinea pig precipitate was tested by the Waaler-Rose and latex techniques, no demonstrable decrease in titer was observed. In contrast to this, all activity had disappeared when sheep cells sensitized with guinea pig amboceptor instead of rabbit amboceptor were used as detector system.

As previously described, the guinea pig diphtheria antitoxin was a pool of 29 individual sera. It had been found that each of the ten individual guinea pig amboceptors studied was, at least to some degree, capable of differentiating rheumatoid sera from normal ones. Thus, it seems reasonable to assume that precipitating systems in which the antibody portion is of guinea pig origin also behave similarly to the amboceptor—sheep erythrocyte complex.

Attempts were made to immunize guinea pigs with Ea and to test the reactivity of every immune serum separately, but the antibody response was not sufficient.

Absorption with a large amount of diphtheria toxoid—horse antitoxin precipitate left the RF activity intact. No decrease in titer could be observed even when using sheep cells sensitized with horse amboceptor.

In a previous paper (3) it has been described that good recovery of RF activity as measured with the Waaler-Rose, latex and SHC

techniques was obtained by eluating human antitoxic precipitates treated with RF.

In the following experiments, attempts were made to compare the nature of RF eluated from precipitates when a large excess or a moderate amount of RF was subjected to absorption. It was assumed than when a large amount of RF is available, selection takes place and only the most avid molecules are adsorbed onto the precipitates. (The possibility that all the RF in a very high-titered rheumatoid serum is of the same avid quality can be excluded on the basis of absorption experiments described on page 27. No definite reduction in the SHC and latex titers was observed after absorption of the Waaler-Rose activity from the serum K.O.)

First, two 1.5 ml portions of the undiluted exceptionally high-titered rheumatoid serum K.O. (Waaler-Rose titer 8192, latex titer $\geq$ 327680, and SHC Ripley titer $\geq$10240) were absorbed with 310 μg N of human antitoxic precipitate H.K. and 173 μg N of Ea—rabbit anti-Ea precipitate, respectively. Only a very small decrease, ranging from 0 to 2 tubes, was seen in the titer values. After thorough washing the precipitates were eluated. The RF activity of the eluates is shown in table 8.

It can be seen from table 8 that RF activity measurable by the Waaler-Rose, latex, and SHC (Ripley) techniques was contained in both eluates. The residue of each eluate was divided into two

TABLE 8

REACTIVITY OF RF ELUATED FROM SPECIFIC PRECIPITATES TREATED WITH A HIGH-TITERED RHEUMATOID SERUM

	Eluate Made from Human Antitoxic Precipitate H.K. Treated with Rheumatoid Serum K.O.			Eluate Made from Rabbit Anti-Ea Precipitate Treated with Rheumatoid Serum K.O.		
	Waaler-Rose	Latex	SHC (Ripley)	Waaler-Rose	Latex	SHC (Ripley)
Non-absorbed eluate	2560	10240	2560	2560	10240	1280
Eluate absorbed with human antitoxic precipitate	<16	<40	<40	<16	<40	<40
Eluate absorbed with rabbit anti-Ea precipitate	<16	<40	<40	<16	<40	<40

equal parts. One part was absorbed for two hours with 310 μg N of human antitoxic precipitate H.K. and the other part with 173 μg N of Ea—rabbit anti-Ea precipitate for the same time. It can be seen from table 8 that this absorption resulted in total disappearance of RF activity in all of the four combinations and in the three tests used.

Next, another rheumatoid serum T.A. with lower RF activity (Waaler-Rose titer 1024, latex titer 20480, and SHC Ripley titer 10240) was absorbed with the same amounts of human and rabbit precipitates as in the previous experiment. This particular serum, in contrast to serum K.O., was also capable of agglutinating O Rh positive cells sensitized with anti-D 201/60 up to titer 2560. After absorption with rabbit precipitate, all Waaler-Rose activity had disappeared, whereas SHC (Ripley), SHC (201/60) and latex activities remained nearly unaltered. On the other hand, absorption with human precipitate removed practically all of the SHC (201/60) activity without affecting significantly the three other varieties of RF activity.

Titer determinations from the eluates are shown in table 9. As in the preceding experiment, both eluates contained RF activity

TABLE 9

REACTIVITY OF RF ELUATED FROM SPECIFIC PRECIPITATES TREATED WITH A MEDIUM-TITERED RHEUMATOID SERUM

	Eluate Made from Human Antitoxic Precipitate H.K. Treated with Rheumatoid Serum T.A.			
	Waaler-Rose	Latex	SHC	
			(Ripley)	(201/60)
Non-absorbed eluate	256	2560	2560	640
Eluate absorbed with rabbit anti-Ea precipitate	<16	not done		160
	Eluate Made from Rabbit Anti-Ea Precipitate Treated with Rheumatoid Serum T.A.			
	Waaler-Rose	Latex	SHC	
			(Ripley)	(201/60)
Non-absorbed eluate	128	2560	320	<20
Eluate absorbed with rabbit anti-Ea precipitate	<16	not done		<20

measurable by the same three techniques as were used above. In SHC (201/60) test, however, no activity was demonstrable in the eluate made from rabbit precipitate. Absorption with a large amount (346 μg N) of rabbit precipitate of eluate made from human precipitate resulted in only a small reduction in the SHC (201/60) titer.

Taking into consideration the poor absorptive capacity of guinea pig precipitate, poor results in elution experiments also were to be expected. The R-pool and the rheumatoid serum S.K. (Waaler-Rose titer 1024, latex titer 40960, and SHC Ripley titer 10240) were used. Experiments were carried out using 95 μg N of specific precipitate per milliliter of serum. In the eluate from serum S.K. there were demonstrable a Waaler-Rose titer of 32, latex titer of 320 and SHC (Ripley) titer of 80, expressed in terms of the original serum volume. R-pool gave still less activity. Very weak reaction was obtained when the eluates were tested with sheep cells sensitized with guinea pig amboceptor.

C. SPECIFICITY OF RHEUMATOID FACTOR ADSORBED ONTO AND ELUATED FROM SENSITIZED CELLS

Rheumatoid sera V.I. and T.A. were diluted 1 : 10. One part of the serum dilution was added to one part of packed sheep erythrocytes sensitized with homologous rabbit amboceptor corresponding to 2 MAD. After overnight in the refrigerator the serum dilution was separated and transferred to a fresh portion of sensitized cells. The first portion of cells was washed and used for elution experiments. After the second absorption the serum dilution was tested for RF activity using the Waaler-Rose and SHC (201/60) tests. The Waaler-Rose test using cells sensitized up to ½ MAD was completely negative. On the other hand, no measurable titer decrease was observed in the SHC (201/60) test.

The elution was tried by keeping the cells for one hour at 45°C. The Waaler-Rose made from the eluate was completely negative. Weak agglutination up to titer 640 was demonstrable in the latex test. The SHC (201/60) test was negative, whereas anti-D Ripley serum and rheumatoid serum T.A. (which latter had higher titers than V.I.) showed agglutination in the supernatant up to titer 80. The use of greater amounts of RF in adsorption resulted in demonstrable Waaler-Rose activity in eluates.

Shortage of anti-D Ripley and 201/60 prevented their use in the absorption of RF. Therefore, absorption experiments were attempted using O Rh positive cells sensitized with three anti-D sera which were available in sufficient amounts. O Rh positive cells sensitized with these sera gave weak agglutination reactions with selected rheumatoid arthritis sera. One part of packed cells and two parts of the anti-D serum in question in ten parts of saline for coating the cells were used. One part of rheumatoid serum T.A. in dilution 1 : 20 was absorbed three times with one part of packed cells. In two instances this resulted in complete disappearance of agglutination of O Rh positive cells sensitized with anti-D 201/60 and in one instance in a markedly reduced titer. The Waaler-Rose titers were unaltered. Absorption with a twofold amount of sensitized cells did not appreciably reduce the SHC (Ripley) titer.

Absorption of the rheumatoid serum with sheep cells sensitized with homologous horse anti-sheep amboceptor resulted in disappearance of the agglutinating activity for sheep cells sensitized with horse amboceptor. The Waaler-Rose titer remained unaltered.

DISCUSSION AND CONCLUSIONS

In table 3 it was shown that the agglutination of sensitized O Rh positive cells takes place at higher temperatures than the Waaler-Rose reaction. Fraser (16) in his studies of T agglutinin used absorptions at various temperatures for differentiating normal from immune agglutinins. The more avid immune agglutinins were absorbable at higher temperatures than the less avid normal ones. Thus, the results obtained may be interpreted on the basis of a more avid bond between the RF and the reactant of human origin than that of rabbit origin.

The finding that the RF reacts better with Ea—rabbit anti-Ea precipitate made in antigen excess than with that made in antibody excess is in accordance with the observation of Edelman *et al.* (13). However, their technique differed from that used in the present work since they added the RF preparation to the antigen-antibody mixture and not to the pre-formed precipitate. Thus, the nitrogen increase observed by them was due in part to soluble antigen-antibody complexes precipitated by the RF.

As there evidently are more antigen-antibody bonds in the region of slight antigen excess than of antibody excess, the results obtained support the hypothesis that the reaction of the RF takes place with antigen-antibody complexes rather than with unaltered gamma globulin.

The experiments carried out with human antitoxic precipitates give rise to somewhat conflicting conclusions. It was difficult to absorb RF activity away from the whole serum with rheumatoid antitoxic precipitates, whereas the absorption was readily accomplished from redissolved cold precipitates made of rheumatoid sera. These results suggest an equilibrium reaction between the RF and the reactant existing bound in the antibody portion of the precipitate and free in the surrounding human serum milieu. Furthermore, this absorption pattern indicates that there is no great difference between the reactants in the antitoxic precipitate and in the serum with respect to their affinity for the RF, provided that the RF remaining in the supernatant does not differ qualitatively from that in the cold precipitate.

Harboe (23) independently has confirmed and to some degree extended the observations described in the present report by demonstrating that also non-rheumatoid human specific precipitates (varidase—anti-varidase) interact with the RF.

It was shown that diphtheria toxoid—guinea pig antitoxin precipitate wholly absorbed the RF activity measurable with sheep cells sensitized with guinea pig amboceptor. The corresponding agglutination reaction with rabbit amboceptor remained practically unaltered. On the other hand, only a part of the RF measurable with guinea pig reactant was adsorbable onto rabbit precipitate.

Horse diphtheria antitoxin precipitate wholly failed to react with the RF. No experiments were made to study whether the antitoxin might be of 19 S variety.

A general pattern emerges from the absorption experiments described in this chapter. Precipitates with an antibody portion of human origin [1] are capable of completely absorbing all the RF activity measurable with any detector system. Precipitates with

[1] Both of the human antitoxic sera were of the inhibitory type, Gm (a+). No antitoxic sera of the type Gm (a—) were available. It has been observed, however, that, at least, heat-denatured gamma globulin preparations deriving from a number of rheumatoid or non-rheumatoid subjects do not markedly differ in their capacity to interact with the RF (29).

an antibody portion from other species, if reactive, absorb completely only the activity measurable with an isogenous reactant. This high specificity of the absorption pattern of the RF is the best proof excluding the possibility of non-specific aggregation of the factor.

Absorption experiments with sensitized cells led to results similar to those obtained with specific precipitates, with the following exceptions. First, horse amboceptor was suitable for demonstrating RF activity, whereas horse diphtheria antitoxin precipitate did not absorb it, and secondly, absorption with O Rh positive cells sensitized with certain incomplete anti-D sera removed only a small fraction of the total RF activity of the serum.

The reactivity patterns of the RF after absorption with different antigen-antibody complexes are summarized in table 10.

TABLE 10

THE REACTIVITY PATTERNS OF THE RF AFTER ABSORPTION WITH DIFFERENT ANTIGEN-ANTIBODY COMPLEXES

Source of Antibody Used for Absorption		RF Activity in the Supernatant after Absorption with Antibody from				
		Human		Rabbit	Guinea Pig	Horse
		Ripley	Gm (a)			
Human in	specific precipitate	—	—	—	—	—
	sensitized cells	+	—	+	not done	
Rabbit in	specific precipitate	+	+	—	+	+
	sensitized cells	+	+	—	not done	
Guinea pig in	specific precipitate	+	+	+	—	+
	sensitized cells	not done				
Horse in	specific precipitate	+	+	+	+	+
	sensitized cells	not done		+	+	—

Elution experiments gave some additional information on the specificity of the RF. Since strong activity was demonstrable in the SHC (Ripley) test in the eluate made from rabbit precipitate (table 8), the reactive groupings responsible for agglutination of sheep cells sensitized with rabbit amboceptor and of O Rh positive cells sensitized with anti-D Ripley are present at least in part in the same molecules. On the other hand, it is apparent from the experiments described in table 9 that the reactive groupings

responsible for the positive Waaler-Rose reaction and for the agglutination of O Rh positive cells sensitized with anti-D 201/60 (anti-Gm (a) activity) do not exist in the same molecules or do so to very small degree only (at least in the particular serum used in these experiments).

Further it was observed that all the RF activity eluated from human precipitate treated with a large excess of RF was adsorbable onto the rabbit precipitate, and *vice versa* (table 8). Thus, it appears that the characteristic feature of »avid» RF is a broad cross-species reactivity. In this respect »avid» RF differs from RF in whole serum or in eluate made from precipitates treated with small amounts of RF.

SUMMARY

The agglutination by the rheumatoid factor (RF) of O Rh positive cells sensitized with anti-D sera (SHC activity) took place at high temperatures that already inhibited the agglutination of sheep cells sensitized with rabbit amboceptor (Waaler-Rose activity).

Differential absorption studies performed with specific precipitates showed that human diphtheria antitoxin precipitates completely absorbed the RF activity measurable with any of the detector systems studied. Absorption with precipitates containing antibody from other species, however, gave a different result, completely removing only the RF activity measurable with the isogenous reactant—amboceptor system.

Absorption and elution experiments suggested that the reactive groupings responsible for a positive Waaler-Rose reaction and for agglutination of O Rh positive cells sensitized with anti-D serum suitable for Gm (a) typing do not exist in the same molecules. This, however, seems to be the case with some of the groupings responsible for the Waaler-Rose and the SHC (Ripley) activities, as suggested by the presence of SHC (Ripley) activity in eluates made from rabbit immune precipitates treated with RF.

The RF molecules of the highest »avidity» appeared to possess the greatest cross species reactivity.

CHAPTER V

INHIBITION OF THE RHEUMATOID FACTOR BY GAMMA GLOBULINS

Winblad (51) observed that diluted rabbit serum inhibited the Waaler-Rose activity. This inhibition phenomenon was more clearly seen when weakly sensitized cells were used. Subsequently Heller and coworkers (26) showed that some human sera possessed a similar effect. The inhibitory substance was concentrated in Cohn fraction II containing mainly gamma globulin. It has been postulated that the rheumatoid factor possesses affinity for gamma globulin, the inhibition being due to a competition for the RF between the sensitizing antibody and the inhibitory gamma globulin. Despite demonstration of reactant in the isolated fraction II of patients with RF in their circulation (29), the euglobulin fraction of rheumatoid sera failed to inhibit the Waaler-Rose activity, whereas euglobulin fractions of normal sera showed an inhibitory effect (54).

Kunkel (28) observed that human gamma globulin preparations become highly inhibitory when heated at 63°C for ten minutes. Such heated gamma globulin readily inhibited the agglutination of sensitized sheep cells (Waaler-Rose), agglutination of sensitized human cells (SHC), and agglutination of F II-treated tanned erythrocytes (41).

Evidently another variety of inhibitor was demonstrated by Grubb (21). He found that selected rheumatoid sera agglutinate O Rh positive cells sensitized with certain incomplete anti-D sera and that some human sera inhibit this agglutination. The inhibitory capacity was shown to be a hereditary characteristic (22, 24).

In order to complete the absorption and elution experiments described in the previous chapter, inhibition experiments were

made to demonstrate the interaction of the RF and soluble reactants, and particularly to correlate the relative inhibitory capacity of various gamma globulin preparations. Furthermore, inhibition experiments were carried out with rabbit anti-RF immune sera.

METHODS

Inhibition Experiments. — One part of diluted rheumatoid serum and one part of diluted serum or protein preparation to be tested for its inhibitory capacity were measured into each tube. After 15 minutes at room temperature, two parts of sensitized cells were added into each tube. A check-board titration was always performed by making a dilution series both of the rheumatoid serum and of the inhibitory substance under test.

Inhibition experiments of the Waaler-Rose activity were made using a pooled rheumatoid serum (R-pool). The cells were usually sensitized with $^1/_2$, $^1/_4$ and $^1/_8$ MAD. The total volume of the reaction mixture was 1 ml. The results were read on the following day against the light of a desk lamp and as bottom readings against the even light of a mercury vapor lamp.

The total volume of reagents in inhibition experiments of the SHC test (and also in inhibition experiments of the Coombs test in chapter VI) was 0.2 ml. The results were read microscopically after one hour. If not otherwise stated, rheumatoid sera V.I. and T.A. and anti-D sera 201/60 and Ripley were used in all four combinations.

The expressed dilutions and amounts of inhibitory substances always were calculated from the total volume of reagents in the tube. The relative inhibitory capacities of substances were estimated on the basis of the highest dilution which still inhibited a given amount of RF activity under appropriate conditions.

Production of Anti-RF Serum. — Four rabbits (Nos. 150—153) were immunized. Two of the total of 30 injections were given subcutaneously with Freund incomplete adjuvant and the others intravenously with alumina gel. Each rabbit received in all during 12 weeks about 0.25 mg of RF nitrogen adsorbed onto 2.5 mg of specific precipitate nitrogen. The rabbits were bled two weeks after the last injection.

The determinations of the gamma globulin content of sera were made by N. Saris, Lic. Phil. The paper electrophoresis technique and a modification of the biuret method were used.

RESULTS

Inhibition of the Waaler-Rose activity (R-pool, cells sensitized with ½ MAD) by pooled human serum, human gamma globulin, and human gamma globulin heated for 10 minutes at 63°C is

TABLE 11

INHIBITION OF THE WAALER-ROSE ACTIVITY BY POOLED HUMAN SERUM, HGG, AND HEATED HGG

μg N per ml of Inhibitory Gamma Globulin	Waaler-Rose Titer [1] of R-pool when the Inhibitory Agent was		
	Pooled Serum	HGG	Heated HGG
50	1024	512	<32
25	1024	512	32
12	1024	512	32
6	1024	1024	64
3	1024	1024	128
1.5	1024	1024	128
None	1024	1024	1024

[1] The sheep cells were sensitized with ½ MAD of rabbit amboceptor.

shown in table 11. The results given were read by the bottom reading technique.

As seen in table 11, pooled human serum exerted no observable inhibitory effect on the Waaler-Rose activity. This same held true even when cells sensitized with $^1/_4$ and $^1/_8$ MAD (not shown in the table) were used. Heated HGG, on the other hand, was an effective inhibitory agent.

Heated or non-heated rabbit or human globulin prepared by ammonium sulphate fractionation did not exert greater inhibition than the corresponding non-fractionated serum.

As shown in table 12, a pooled normal rabbit serum called forth a definite inhibition phenomenon when cells sensitized with $^1/_8$ or $^1/_4$ MAD were used. No clear inhibition was observed when using cells sensitized with ½ MAD. In contrast to this, pooled rabbit immune serum (anti-Ea) brought about marked inhibition even when cells sensitized with ½ MAD were used. The titers shown in table 12 are corrected according to amounts of gamma globulin in the serum.

In order to obtain additional information on the observed difference between normal and immune rabbit sera, the prebleeding and bleeding No. I from six rabbits (Nos. 181—186) immunized with Ea were studied with reference to this point. The immunization schedule has been described elsewhere (4). In the immune bleedings of three rabbits (Nos. 182, 185 and 186) there were »anti-

TABLE 12

INHIBITION OF THE WAALER-ROSE ACTIVITY BY POOLED NORMAL AND IMMUNE RABBIT SERA

Dilution of the Inhibitory Serum	Cells Sensitized with	Waaler-Rose Titer [1] when the Inhibitory Serum was of	
		Normal Type [2]	Immune Type [3]
1/32	1/2 MAD	512	64
	1/4 »	32	<32
	1/8 »	<32	<32
1/64	1/2 MAD	512	128
	1/4 »	64	<32
	1/8 »	<32	<32
1/128	1/2 MAD	512	256
	1/4 »	128	32
	1/8 »	<32	<32
1/256	1/2 MAD	1024	256
	1/4 »	256	64
	1/8 »	64	<32
1/512	1/2 MAD	1024	512
	1/4 »	256	128
	1/8 »	64	32
1/1024	1/2 MAD	1024	1024
	1/4 »	256	128
	1/8 »	64	64
—	1/2 MAD	1024	1024
	1/4 »	256	256
	1/8 »	64	64

[1] The titers in the table were recorded by the bottom reading technique.
[2] A pool of 3 individual rabbit normal sera.
[3] A pool of 8 individual rabbit anti-Ea sera.

antibodies» which agglutinated sheep cells sensitized with rabbit amboceptor. After absorption of the »anti-antibodies» with HGG—rabbit anti-HGG specific precipitate as described elsewhere (4), these sera, also, could be used for inhibition experiments.

Sheep erythrocytes were sensitized with 1/3 MAD alone. In table 13 are shown the results from these sera.

The prebleedings from rabbits 183 and 184 called forth a definite inhibition, and immunization did not increase it. In contrast to this, a marked change from non-inhibitory to inhibitory had taken place in the other four sera. The approximate antibody con-

TABLE 13

INHIBITION OF THE WAALER-ROSE ACTIVITY BY PREIMMUNE AND IMMUNE BLEEDINGS FROM SIX INDIVIDUAL RABBITS

Rabbit No.	Type of Bleeding	Dilution of Pooled Rheumatoid Serum (R-pool)							Anti-Ea Antibody Content (μg N/ml.)
		32	64	128	256	512	1024	2048	
181	preimmune	+++	+++	+++	++	+	—	—	500
	immune	++	++	+	(+)	—	—	—	
182	preimmune	+++	+++	+++	++	+	—	—	250
	immune	+	+	—	—	—	—	—	
183	preimmune	++	++	—	—	—	—	—	500
	immune	++	+	—	—	—	—	—	
184	preimmune	+++	+++	++	+	—	—	—	250
	immune	+++	+++	+	+	—	—	—	
185	preimmune	+++	+++	++	++	+	—	—	125
	immune	+	+	—	—	—	—	—	
186	preimmune	++	+++	++	++	+	(+)	—	500
	immune	—	—	—	—	—	—	—	
No inhibitor		+++	+++	++	++	+	(+)	—	

The inhibitory sera were diluted 1/32.

tent of these sera was estimated as described on page 22. As shown in table 13, the amount of precipitable antibodies did not correlate with the inhibitory capacity of the serum in question.

Guinea pig serum and bovine gamma globulin (lot DC 1473 of Armour Pharmaceutical Co., Eastbourne, England), heated or non-heated, did not exert an appreciable inhibitory effect.

Next, inhibition experiments with specific precipitates were carried out. It was shown that Ea—rabbit anti-Ea and diphtheria toxoid—rabbit antitoxin precipitates inhibited the Waaler-Rose activity 50—100 times better than pooled immune rabbit serum and about 10 times better than heated HGG if calculated per μg N of gamma globulin. Further it was observed that the supernatant of Ea—rabbit anti-Ea precipitate made in slight antibody excess was inhibitory to the same degree as the pooled anti-Ea serum from which the precipitate originated. Absorption of three normal rabbit sera with the same precipitate did not appreciably increase their inhibitory capacity.

Thereafter, the inhibition of Waaler-Rose activity produced by rabbit anti-RF serum was studied. In immunoelectrophoresis

carried out against a human serum with elevated immunoglobulins it was shown that each of the four anti-RF sera contained precipitins for gamma globulin and for beta$_2$ M globulin, and small amounts of precipitins for some other antigens belonging to the beta globulin fraction. Due to the antibodies to the gamma fraction, the anti-RF sera agglutinated sensitized O Rh positive cells up to log$_2$ titer of 10.

In order to avoid antigen excess and soluble antigen-antibody complexes, the anti-RF sera were absorbed with O Rh positive cells sensitized with incomplete anti-D antibodies, instead of employing gamma globulin solution for removal of the antibodies for gamma globulin (46),

The inhibitory effect of the pooled anti-RF serum for the Waaler-Rose activity is shown in table 14.

TABLE 14

INHIBITORY EFFECT OF RABBIT ANTI-RF SERUM ON THE WAALER-ROSE ACTIVITY

Dilution of the Inhibitory Serum	Waaler-Rose Titer [1] of Pooled Rheumatoid Serum Inhibited by	
	Non-Absorbed Anti-RF Serum	Absorbed [2] Anti-RF Serum
1/32	<32	<32
1/64	<32	32
1/128	64	64
1/256	128	128
1/512	256	256
1/1024	256	256
1/2048	512	512
No inhibitor	1024	1024

[1] Sheep cells sensitized with ½ MAD of rabbit amboceptor were used.
[2] Absorbed with O Rh positive cells sensitized with incomplete anti-D serum.

It can be seen that the anti-RF serum exerted a marked inhibitory effect. Further it is shown in the table that after absorption of the anti-gamma globulin antibodies the inhibitory effect was unaltered. One of the four sera, No. 150, tested separately under identical conditions, gave a similar inhibition.

Next, the SHC activity was subjected to inhibition studies. The relative inhibitory capacity of pooled human serum, HGG and heated HGG on the SHC activity, calculated per agglutinating

unit (the use of anti-D Ripley resulted in four times as high titers as the use of anti-D 201/60), is shown in table 15. The values given in the table represent average values in the range of 16—64 agglutinating units.

TABLE 15

INHIBITION OF THE SHC ACTIVITY BY POOLED HUMAN SERUM, HGG, AND HEATED HGG

Inhibitory Agent	Log_2 of the Relative Inhibitory Capacity when the Cells were Sensitized with	
	Anti-D Ripley	Anti-D 201/60
Pooled human serum	1	4
HGG	2	4—5
Heated HGG	5—6	5—6

As illustrated in table 15, heated HGG inhibited equally effectively the agglutination of cells sensitized with anti-D Ripley and anti-D 201/60. The latter agglutination was about 8 times as readily inhibitable by normal human serum and non-heated gamma globulin than the former agglutination.

No inhibition of the SHC activity by bovine gamma globulin was observed. Normal rabbit serum and Ea—rabbit anti-Ea precipitate caused a weak inhibition, about 50—100 times as weak as that given by pooled human serum.

TABLE 16

INHIBITORY EFFECT OF RABBIT ANTI-RF SERUM ON AGGLUTINATION OF SENSITIZED HUMAN CELLS (SHC)

Dilution of the Inhibitory Serum	Agglutination Titer for SHC [1] of Serum T.A. Inhibited by	
	Anti-RF Serum Absorbed with SHC [2]	Pooled Normal Rabbit Serum
1/8	40	160
1/16	80	320
1/32	160	640
1/64	320	640
1/128	640	1280
1/256	640	1280
No inhibitor	1280	1280

[1] O Rh positive cells sensitized with anti-D 201/60.
[2] O Rh positive cells sensitized with anti-D Ru.

The inhibition of SHC activity by anti-RF serum is shown in table 16. The anti-RF serum was absorbed with sensitized O Rh positive cells as in the inhibition experiments of the Waaler-Rose activity. Only rheumatoid serum T.A. and anti-D serum 201/60 were used.

It can be seen that the anti-RF serum inhibited the SHC activity to some degree, but considerably less than the Waaler-Rose activity (cf. table 14).

DISCUSSION AND CONCLUSIONS

The experiments illustrated in table 11 showed that the Waaler-Rose activity of pooled rheumatoid serum was not inhibitable by pooled normal human serum, whereas heated HGG was an effective inhibitory agent. It has been shown previously (26, 39) that some human sera markedly inhibit the Waaler-Rose activity of individual sera, whereas other human sera exert no inhibition or do so to a very small degree only. The failure to demonstrate inhibition in the author's experiments carried out with pooled rheumatoid serum might have been due to the circumstance that the RF activity of some rheumatoid sera is not inhibitable by any human serum. The effect of heating is difficult to explain otherwise than that it exposes groupings which are hidden in native HGG and are similar to those in the sensitizing amboceptor.

It can be postulated that some reactive groupings in the rabbit amboceptor are poorly represented in normal rabbit gamma globulin and that efficient immunization, irrespective of the antigen used, favors the production of gamma globulin suitable for the competition with the sensitizing amboceptor. The increased inhibitory effect of rabbit serum due to immunization cannot be explained solely by an increase of gamma globulin in the rabbit serum. It is also unlikely that the phenomenon was due to soluble antigen-antibody complexes, because the supernatant of the rabbit anti-Ea precipitate made in slight antibody excess was inhibitory to the same degree as the original immune serum. (The complexes most likely would have been co-precipitated.)

On the other hand, the agglutination of O Rh positive cells sensitized with anti-D suitable for Gm typing (201/60) was readily inhibited by pooled human serum. The fractionation procedure

and heating of the gamma globulin fraction did not appreciably increase the inhibitory effect. In contrast to this, the agglutination mediated by anti-D Ripley was more readily inhibited by heated than by non-heated HGG. Thus it appears that denaturation of the reactant does not play a marked role in the reaction between the RF and the anti-D serum suitable for Gm typing, whereas it does so to some degree in the reaction between the RF and the anti-D Ripley (cf. also tables 8 and 9).

The significance of these inhibition experiments is further discussed on page 50.

In tables 14 and 16 the inhibitory effect of rabbit anti-RF serum on the Waaler-Rose and SHC activities is shown. If we omit the not plausible explanation that the whole inhibition of the Waaler-Rose activity was »non-specific», i.e., due to the immune serum effect, it appears that the anti-RF serum inhibits better the Waaler-Rose activity than the SHC activity. It is not reasonable to assume that the rabbit can distinguish antigenic differences between various reactive components of the RF, as no antigenic differences have been found to exist even between the RF and the normal beta$_2$ M-globulin. A possible explanation for the observed difference is that the RF may have greater affinity to the reactant of human origin than to that of rabbit origin.

SUMMARY

The Waaler-Rose activity of pooled rheumatoid serum was not inhibitable by pooled normal serum whereas heated HGG was an effective inhibitory agent. It was also inhibitable by normal and, still better, by immune rabbit serum.

The SHC activity was inhibitable by pooled human serum, whereas normal rabbit serum or rabbit specific precipitates did not appreciably inhibit it. SHC (Ripley) activity was better inhibitable by heated than by non-heated HGG. This difference was not observable when using anti-D serum suitable for Gm typing.

Rabbit anti-RF serum inhibited better the Waaler-Rose activity than the SHC activity.

CHAPTER VI

CROSS REACTION OF GAMMA GLOBULINS FROM VARIOUS SPECIES

Using quantitative immunochemical techniques, Maurer (31) showed that bovine gamma globulin precipitated about 25 per cent of the rabbit anti-human gamma globulin antibody. In the reciprocal cross reaction, human gamma globulin was also capable of removing about 25 per cent of the rabbit anti-bovine gamma globulin. The cross reaction between human and rabbit gamma globulin was to some degree lower.

The anti-globulin (Coombs) inhibition test has been used for determination of small amounts of gamma globulin. Grubb (20) found that albumin or alpha globulin purified by zone electrophoresis did not inhibit the agglutination, whereas beta globulin was inhibitory, but markedly less so than gamma globulin. Fetal gamma globulin was less inhibitory than the adult one. Rabbit and horse sera did not appreciably inhibit the reaction.

Recently Weiler *et al.* (50) have described a labeled antigen precipitation inhibition test for measuring gamma globulin. This method is based on the same principle of inhibition as, but is more precise than, the anti-globulin inhibition test.

The working hypothesis of the author was that there exist two varieties of cross reaction. The first variety can be expressed by the symbols AB and A and the second one by the symbols AB and A_1B_1, where the letters indicate groups of antigenic determinants. The two types, of course, can be mixed: for example, AB and A_1.

It would be expected that antigen A removes a part, and only a part, of the antibodies directed to AB. Thus agglutination of AB by a mixture of anti-A and anti-B molecules is not inhibited by A. The »avidity» of anti-A for AB is the same as the avidity of anti-AB.

It is more difficult to predict the behavior of the AB—A_1B_1 cross reaction. Probably large amounts of A_1B_1 can inhibit the agglutination by anti-AB, and it may be expected that the »avidity» of anti-A_1B_1 to AB is less than that of anti-AB.

Bearing this concept in mind, precipitation, agglutination and agglutination inhibition experiments were carried out with rabbit anti-HGG and rabbit anti-BGG sera to determine the nature of the cross reaction between human and bovine gamma globulins.

MATERIAL AND METHODS

Anti-HGG Serum. — The immunization schedules for production of anti-human gamma globulin sera have been described elsewhere (4). With the micro-immunoelectrophoresis technique (36) one of the 16 anti-HGG sera (No. 139) showed antibodies practically only to gamma globulin. This serum has been used in the experiments described in this chapter. In all other sera there also were antibodies against other serum proteins, in most instances against various beta globulins. In some sera the antibodies to impurities much exceeded the amount of anti-gamma globulin.

Anti-BGG Serum. — Two rabbits (Nos. 179 and 180) were given 16 intravenous injections of alum-precipitated bovine gamma globulin (lot DC 1473 of Armour Pharmaceutical Co., Eastbourne, England). The total amount was 20 mg of protein. Injections were given three times a week. Because this did not result in sufficient antibody response, 8 additional injections were given. The rabbits were bled four days after the last injection.

The antiglobulin (Coombs) test was made in a manner similar to the SHC test described in chapter III. For sensitization of the cells two potent incomplete anti-D sera (Ta and Li), not suitable for rheumatoid agglutination, were used. The role of the anti-globulin serum in the Coombs test was similar to that of the rheumatoid serum in the SHC test.

The anti-globulin inhibition technique was the same as the inhibition technique for the SHC reaction.

The methods of preparing the specific precipitates and performing the nitrogen analyses are described in the technical section of chapter IV. The nitrogen determinations were not made in duplicate.

RESULTS

The cross reaction of anti-human gamma globulin serum with bovine gamma globulin is shown in table 17. It can be seen from this table that the BGG precipitated about 7 per cent of the anti-

TABLE 17

PRECIPITIN REACTION OF RABBIT ANTI-HGG SERUM 139 WITH HGG AND BGG

HGG N Added	Total N Precipitated	BGG N Added	Total N Precipitated
μg	μg	μg	μg
5	43.5		
10	76.0	10	10.0
15	109.0		
20	131.5	20	15.0
30	175.5	30	16.0
40	222.5	40	16.5
60	296.5	60	19.5
80	251.5	80	17.5
100	257.0	100	13.5
120	242.0	120	18.0

All values stated per ml of serum dilution used. Serum 139 diluted 1:2.

bodies to HGG. Attention is also drawn to the poor inhibition of precipitation by anti-HGG serum in the region of antigen excess.

The reciprocal cross reaction between anti-BGG serum and HGG is demonstrated in table 18. It is observed that the HGG precipitated about 10 per cent of antibodies to BGG.

TABLE 18

PRECIPITIN REACTION OF RABBIT ANTI-BGG SERUM 179 WITH BGG AND HGG

BGG N Added	Total N Precipitated	HGG N Added	Total N Precipitated
μg	μg	μg	μg
10	108.0	10	28.0
20	183.5	20	28.0
30	237.5	30	27.0
40	262.5	40	28.0
60	293.5	60	29.5
80	284.0	80	34.0
100	265.5	100	25.0

All values stated per ml of serum dilution used. Serum 179 diluted 1:2.

Next, the capacity of anti-BGG serum to agglutinate O Rh positive cells sensitized with incomplete anti-D antibodies was studied. Some of the results are shown in table 19, from which it can be seen that an anti-BGG serum agglutinated O Rh positive cells. The titer values obtained were in good agreement with amounts of precipitating antibodies shown in table 17. Another

anti-BGG serum (No. 180) behaved in a similar manner. There also were no differences in behavior between the two anti-D sera used for sensitization.

TABLE 19

CAPACITY OF RABBIT ANTI-HGG (139) AND ANTI-BGG (179) SERA TO AGGLUTINATE O RH POSITIVE CELLS SENSITIZED WITH ANTI-D LI SERUM

Serum	Volume of		Agglutination Titer
	Packed Cells	Sensitizing Anti-D Serum	
Anti-HGG	1	4	32768
	1	1	32768
	1	¼	8192 [1]
	1	none	64
Anti-BGG	1	4	4096
	1	1	8192
	1	¼	512 [1]
	1	none	16

[1] Weak agglutination pattern.

Agglutination experiments with anti-human and anti-bovine gamma globulin sera (Nos. 139 and 179) were carried out as described on page 24. No differences between the agglutination patterns of these sera were observed at different temperatures.

TABLE 20

INHIBITION BY HGG AND BGG OF THE COOMBS ACTIVITY OF ANTI-HGG SERUM

Inhibitory Gamma Globulin	Volume of		Dilution of Anti-HGG Serum 139	μg N per ml of Gamma Globulin Giving Complete Aggl. Inhibition
	Packed Cells	Sensitizing Anti-D Serum		
HGG	1	4	1/400	6
	1	1		6
	1	¼		1.6
HGG	1	4	1/1600	0.8
	1	1		0.8
	1	¼		0.4
BGG	1	4	1/400	>100 [1]
	1	1		>100
	1	¼		>100
BGG	1	4	1/1600	>100
	1	1		>100
	1	¼		>100

[1] No agglutination was seen in the control tube with non-sensitized cells.

Representative examples of inhibition experiments are shown in tables 20 and 21. It can be observed from table 20 that bovine gamma globulin exerted no inhibition whatsoever on the agglutination by anti-HGG serum. In contrast to this, as shown in table 21, HGG inhibited the agglutination by anti-BGG serum. Calculated per μg N, the inhibition was even better than that brought about by BGG or bovine serum.

TABLE 21

INHIBITION BY HGG AND BGG OF THE COOMBS ACTIVITY OF RABBIT ANTI-BGG SERUM

Inhibitory Gamma Globulin	Anti-BGG Serum	μg N per ml of Inhibitor						
		4	2	1	½	¼	1/8	0
BGG	179	—	—	(+)	+	++	+++	+++
HGG	179	—	—	—	—	+	++	+++
BGG	180	—	—	—	+	++	+++	+++
HGG	180	—	—	—	—	—	+	+++

Dilution of anti-BGG serum was 1/400.
Equal parts of packed cells and anti-D Li were used for sensitization.

DISCUSSION AND CONCLUSIONS

The results of the experiments described in this chapter fulfill the postulated preconception concerning the cross reaction of type AB—A. If anti-B alone can agglutinate cells sensitized with AB and if anti-A and anti-B are different molecules, it follows that agglutination by an anti-AB serum cannot be inhibited by A irrespective of the amounts of A used. Thus, it is impossible on the basis of inhibition experiments alone to draw any conclusions concerning the relative amounts of anti-A and anti-B.

When these results are compared with the behavior of the RF, it does not seem probable that HGG in its native state is the primary reactant partner of the RF. Let us suppose that the RF is an antibody against HGG and that it partly reacts with antigenic determinants which are not available for reaction in the native state of HGG and which are similar to the sensitizing rabbit amboceptor. Further, it is supposed that these determinants become available for reaction when the HGG is denatured by, for example, serological reaction or heating. Thus, on the basis of the fore-

going, no conclusions can be drawn as to the relative portion of RF which is directed only toward the hidden determinants of HGG, although the native HGG does not inhibit the Waaler-Rose activity of the pooled rheumatoid serum.

An interesting observation having no relation to the antibody nature of the RF was the poor inhibition of precipitation by anti-HGG serum No. 139 in the region of antigen excess. It has been shown (8, 9) that the extended region of precipitation in the antigen excess can be due to the presence of antibodies against impurities. Since the immunoelectrophoretic analysis has shown that rabbit serum No. 139 contained precipitating antibodies to gamma globulin only, this cannot be the explanation. Probably the inhomogeneity lies in the gamma globulin itself. Thus, the results obtained bear out the observation of Porter (35) that in prolonged immunization the antibodies produced are directed against an increasing number of potential antigenic sites of a protein antigen.

SUMMARY

Rabbit anti-BGG serum agglutinated O Rh positive cells sensitized with incomplete anti-D sera. The titer value was proportionate to the amount of precipitating antibodies to HGG. This agglutination was readily inhibited by BGG and HGG. In contrast to this, BGG did not inhibit agglutination by rabbit anti-HGG serum.

CHAPTER VII

EXPERIMENTAL PRODUCTION OF »ANTI-ANTIBODIES»

The rheumatoid factor behaves in many respects like an antibody directed to antigen-antibody complexes or to antibodies denatured in a serological reaction. Experiments have been made to produce »anti-antibodies» in rabbits by stimulating them with antigen-antibody complexes (12, 33), but the existence of antigenically different serum groups in rabbits (34) has made the proper interpretation of the results difficult.

Recently Abruzzo and Christian (1) have demonstrated, by injecting rabbits intravenously for prolonged periods with *E. coli* cultured in a medium containing only salts and glucose, the appearance of antibody-like substance similar to the rheumatoid factor. It agglutinated sensitized sheep cells, tanned sheep cells »coated» with aggregated human gamma globulin, and F II latex particles. It precipitated with aggregated human gamma globulin and was absorbable with specific precipitate and with small amounts of *E. coli*.

Independently, the author described the production in rabbits, by means of auto-stimulation with soluble protein antigens, of »anti-antibodies» causing agglutination of sheep cells sensitized with rabbit amboceptor (4). The »anti-antibodies» reacted with isogenous antibodies denatured in serological reaction with the corresponding antigens. They also reacted with the autogenous antibody and were not inhibited by normal rabbit sera. They did not react with flocculating horse diphtheria antitoxin or with human incomplete anti-D antibodies. The »anti-antibodies» resisted heating for 30 minutes at 56°C. They did not require the presence of hemolytic complement for their action.

The work of Abruzzo and Christian prompted the author to further study of the reactivity of the rabbit »anti-antibodies» with human gamma globulin. Some experiments were also carried out to produce human »anti-antibodies».

MATERIAL

Definition of Terms. — According to Coombs and Dubiski (10, 12), auto-stimulation is stimulation with the individual's own (autogenous) antibody denatured in serological reaction with the corresponding antigen.

»Anti-Antibody» Sera. — Rabbit »anti-antibody» sera Nos. 185 and 186 were used. They were produced by auto-stimulation with egg albumin. These same sera were used also in the previous work (4), in which the production of these sera and their characteristics were described.

RESULTS

Rabbit serum No. 185 agglutinated F II latex particles up to titer 640. Serum No. 186 gave a weak agglutination in dilutions 1/160—1/320 with a marked prozone phenomenon. Bovine gamma globulin did not act as a coating agent. (It has been shown in unpublished experiments that Armour BGG was suitable for coating latex particles for rheumatoid agglutination, giving parallel results, though 4—8 times lower titer values, with Kabi HGG). Serum 185 gave a weak precipitin reaction with HGG heated at 63°C for 10 minutes.

The factor agglutinating latex particles was readily absorbable with HGG—rabbit anti-HGG precipitate. This absorption also resulted in disappearance of agglutination of sheep cells sensitized with rabbit amboceptor.

Next, 1 ml of »anti-antibody» serum No. 185 was absorbed twice with 310 μg N of human specific precipitate H.K., which was capable of absorbing RF. Absorption did not affect the agglutination pattern for latex particles or sheep cells sensitized with rabbit amboceptor.

Blood specimens from five non-rheumatoid subjects immunized with diphtheria toxoid were studied. All the five persons were chronically ill aged patients. The immunization schedule was the same as that employed for the patients with rheumatoid arthritis (3) and consisted of six injections given once a week, i.e., a total of about 150 Lf of alum-precipitated toxoid. Following immunization,

the sera of two subjects (E.L. and K.R.) contained more than 200 Lf units of diphtheria antitoxin per milliliter, mainly of the non-precipitating variety. The results of the rheumatoid serological tests made with these blood specimens are shown in table 22. In one instance some increase in the titer values had taken place (E.L.) and in one instance (F.V.) the latex one-tube test had changed from negative to doubtfully positive.

TABLE 22

SEROLOGICAL DATA ON NON-RHEUMATOID SUBJECTS IMMUNIZED WITH DIPHTHERIA TOXOID

Subject	Type of Specimen	Waaler-Rose	One-Tube Latex	Latex Titer	SHC (Ripley)
F.V.	preimmune	<32	—	40	<20
	immune	<32	+?	40	<20
A.L.	preimmune	<32	—	<20	<20
	immune	<32	—	<20	<20
E.L.	preimmune	32	+	1280	20
	immune	128	+	2560	320
K.R.	preimmune	<32	—	<20	<20
	immune	<32	—	20	<20
A.S.	preimmune	<32	—	80	<20
	immune	32	—	40	<20

CONCLUSIONS

By auto-stimulation of rabbits with protein antigens it was possible to produce »anti-antibodies» which reacted with rabbit gamma globulin denatured in a serological reaction. Thus they resembled in many respects the rheumatoid factor. These »anti-antibodies» appeared to cross-react with HGG, as they agglutinated F II latex particles and precipitated heated HGG. The affinity of these »anti-antibodies» to HGG was weak, as shown by the prozone phenomenon and the failure to absorb latex-agglutinating activity with human specific precipitate.

Fairly intensive immunization of non-rheumatoid subjects with diphtheria toxoid did not result in any clearcut »anti-antibody»-like effect.

CHAPTER VIII

GENERAL DISCUSSION

The following circumstances observed in the present work suggest that the rheumatoid factor is primarily reactive with human gamma globulin:

— Those rheumatoid sera which were reactive with rabbit reactant (Waaler-Rose activity) evidently also reacted with human reactants (latex and SHC activities) but not *vice versa.*

— Differential absorption studies with specific precipitates showed that human antitoxic precipitates wholly absorbed the RF activity measurable with any detector system used. Precipitates with the antibody portion from other species (rabbit, guinea pig) completely removed only the activity measurable with isogenous reactant-amboceptor;

— The RF activity measurable with any system was easily inhibited by heated human gamma globulin but not with gamma globulin from other species;

— The agglutination by the rheumatoid factor of O Rh positive cells sensitized with anti-D sera took place at high temperatures that already inhibited the agglutination of sheep cells sensitized with rabbit amboceptor, thus suggesting a more »avid» reaction between the RF and the reactant of human origin (anti-D serum);

— The rabbit anti-RF serum inhibited the Waaler-Rose activity more readily than the SHC activity.

The results obtained in this work do not speak against a possibility that the primary event leading to auto-antigenicity would be a denaturation of the HGG (e.g., in a serological reaction) and that the auto-antibody (RF) secondarily produced would to some degree be cross-reactive with the unaltered HGG. Such a cross reactivity, however, would differ from most of the known patterns

of immunological specificity. If a person of blood group A, for instance, is immunized with AB substance, he produces antibodies only to B, since he is tolerant to A. There is also nothing indicating that the inhibitor in, e.g., the Gm system would be denatured HGG.

Therefore, if one does not accept the afore-mentioned hypothesis, it follows that the primary fault must be sought in the »self—not self» recognition mechanism (5). Possible means leading to the failure of the homeostatic mechanism and to the appearance of a »forbidden clone» might be, e.g., intensive antigenic stimulation or (in animal experiments) the use of mycobacterial adjuvant.

By auto-stimulation of rabbits with protein antigens it was possible to produce »anti-antibodies» which reacted with rabbit gamma globulin denatured in a serological reaction and which cross-reacted slightly with HGG. Whether the primary event leading to the appearance of this rheumatoid factor-like substance really was *in vivo* denaturation of antibody is not quite clear. Thus the animal experiments do not necessarily serve as a model for the anti-antibody hypothesis of the rheumatoid factor.

From the results given by the inhibition experiments (page 41) it appears that the »rheumatoid antibody» is directed partly against antigenic determinants which are hidden in the circulating final gamma globulin molecule. It must be assumed that these hidden configurations are to a large extent similar to those in rabbit gamma globulin denatured in serological reaction and also to some degree to the surface configuration of native rabbit gamma globulin (page 44).

From the general biological point of view, one would expect that any substance molded by the organism to »co-work» primarily with autogenous HGG would behave like the RF, even if it were not necessarily an antibody. The observed association of the greatest cross species reactivity with the greatest »avidity» in the RF molecules (page 36) may be suggestive of the antibody (anti-antibody) nature of the RF. Of the two still open alternatives, »antibody» or »complement-like co-factor» the author prefers the former as the more stimulating working hypothesis for further research.

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